Modeling the Inhibitor Activity and Relative Binding Affinities in Enzyme−Inhibitor-Protein Systems: Application to Developmental Regulation in a PG−PGIP System

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Within a number of classes of hydrolytic enzymes are certain enzymes whose activity is modulated by a specific inhibitor-protein that binds to the enzyme and forms an inactive complex. One unit of a specific inhibitor-protein activity is often defined as the amount necessary to inhibit one unit of its target enzyme by 50 %. No objective quantitative means is available to determine this point of 50 % inhibition in crude systems such as those encountered during purification. Two models were derived: the first model is based on an irreversible binding approximation, and the second, or equilibrium, model is based on reversible binding. The two models were validated using the inhibition data for the polygalacturonase−polygalacturonase-inhibiting protein (PG-PGIP) system. Theory and experimental results indicate that the first model can be used for inhibitor protein activity determination and the second model can be used for inhibitor protein activity determination as well as for comparison of association constants among enzymes and their inhibitor-proteins from multiple sources. The models were used to identify and further clarify the nature of a differential regulation of expression of polygalacturonase-inhibiting protein in developing cantaloupe fruit. These are the first relations that provide for an objective and quantitative determination of inhibitor-protein activity in both pure and crude systems. Application of these models should prove valuable in gaining insights into regulatory mechanisms and enzyme−inhibitor-protein interactions.

Introduction

The interaction of an enzyme from one of several classes of hydrolytic enzymes with its specific inhibitor-protein results in a complex of the two proteins that is inactive or of greatly depressed activity. In many cases, the binding, although strong, is reversible. Examples include polygalacturonases (PG) and polygalacturonase-inhibiting proteins (PGIP) (*1*), pectin methylesterases and pectin methylesterase-inhibiting proteins (*2*), ribonucleases and ribonuclease inhibitors (*3*), and serine proteases with their specific protease inhibitors (*4*). Although binding for the aforementioned enzyme−inhibitor protein systems is highly specific, it can occur either at the active site (*5*) or at a point remote from the active site (*6*). This latter type of interaction location is exemplified by the ribonuclease−ribonuclease inhibitor (*3*) and the PG-PGIP systems (*7*).

Convention often defines one unit of inhibitor-protein activity as the amount of inhibitor required to inhibit one unit of its target enzyme's activity by 50 % (e.g., (*8*)). Enzyme activity frequently must be determined with the use of crude preparations of enzyme, inhibitor-protein, or both, and determination of inhibition is based on measuring a decrease in enzyme activity. In all cases, an objective method for quantifying inhibitor-protein activity in pure or crude extracts does not exist, and certainly there is no existing means to extract information on the mode of interaction between enzyme and inhibitor-protein from activity measurements. We encountered this dilemma while attempting to quantify the level of PGIP activity in crude extracts during purification or in various plant tissues in response to stimuli.

PGIPs from different plants are localized in the plant cell wall, share a basic common structure, and are encoded by a gene family whose expression is stimulated by injury or fungal infection (*9*, *10*). They are glycoproteins of 10−20% carbohydrate on mature polypeptide chains ranging between 300 and 340 amino acid residues (*1*) that are enriched with leucine-rich repeat sequences (*1*, *11*). The interaction of PGIP with PG results in a complex that is inactive or of greatly depressed PG activity. In plant−pathogen interactions, the interaction between plant PGIP and fungal PG is of significant interest as part of the plant's defense system (*12*), as PGIP plays an important role in the prevention of the penetration by pathogenic microorganisms in addition to pectic substance metabolism (*13*, *14*). Because the resistance by plant tissues to infection frequently correlates with PGIP expression and its inhibitory action on fungal PG (*14*, *15*), PGIP is a candidate for genetic engineering to obtain transgenic plants resistant to fungal infection or to impart extended shelf life to fruit (*16*).

Thus, the purpose of this project was to model the interaction between enzymes and their corresponding inhibitor-proteins to provide a valid means to accurately

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quantify inhibitor-protein activity and to derive relations that allow comparison of binding affinities among enzymes and inhibitor-proteins from varied sources. The models derived herein were used to identify and further clarify the nature of a differential regulation of expression of cantaloupe PGIP and can be applied to all other enzyme−inhibitor-protein systems.

Materials and Methods

Reagents. Sodium 4-(2-aminoethylamino)benzenesulfonyl fluoride hydrochloride (AEBSF), ethylenediamine tetraacetic acid (EDTA), bovine serum albumin (BSA), and polygalacturonic acid (PGA) from oranges were purchased from Sigma (St. Louis, MO). The derivatization agent for reducing-group quantification (*17*), 2-cyanoacetamide, was purchased from Acros Organics (Geel, Belgium). All other commonly used buffer salts, acids, and bases were reagent grade.

Source Material for Polygalacturonase (PG) Enzymes. The fungi *Phomopsis cucurbitae*, isolate OK-1062, and *Fusarium solani*, isolate OK-737, were the sources of PG enzymes from cantaloupe fruit pathogens and were obtained from Dr. Benny Bruton, USDA, Lane, OK. *Aspergillus niger* PG was from Sigma (St. Louis, MO) and used without further purification. PG was prepared from fruit lesions produced by each of the fungi by culturing each and then inoculating mature, full-slip cantaloupe fruit according to published procedures (*18*, *19*). Lesions were 6−8 days old when harvested. A crude enzyme extract was prepared from lesions of each organism by grinding the lesion in an equal weight of 2 M NaCl in 0.05 M sodium acetate buffer, pH 5.0. After centrifugation at 10 000 × *g* at 15 °C for 30 min to remove insoluble material, the extract was concentrated ∼20-fold for *P. cucurbitae* and ∼70-fold for *F. solani* on an Amicon YM-10 membrane (Millipore Corp., Bedford, MA). It was subsequently exchanged into 0.05 M sodium acetate buffer, pH 5.0, by diafiltration while in the same apparatus. The diafiltration also facilitated removal of UV-absorbing small molecules that, when not removed, reduced the precision of the enzyme assay.

Preparation of Cantaloupe Fruit PGIP (CmPGIP). Cantaloupe fruit (cv. Magnum 45) for this investigation were from the 2001 crop at the Lane Ag Center, Lane, OK. Female flowers were tagged on the day they opened. Fruit were harvested at 5, 10, 15, 20, 30, and 35 days (full slip) postanthesis. Five-day melons were separated into exocarp, mesocarp, and seeds together with seed cavity material. Fruit at all later stages of development were divided into exocarp, outer mesocarp, mid mesocarp, inner mesocarp, seed cavity (placental material), and seed. Samples were frozen at −20 °C until extracted for PGIP assay.

Tissue samples were extracted by combining a given weight of thawed sample with 1 volume of pre-extraction buffer equal to the tissue sample weight. For mesocarp samples, 52.5 g of tissue was utilized. Samples of 26.25 g were taken for 5-day-old fruit, exocarp, seed, and seed cavity material, and 0.05 M sodium acetate buffer, pH 5.0, containing 1 mM EDTA and 5 μM AEBSF was used as pre-extraction buffer. Samples in pre-extraction buffer were ground in a blender (Waring Products Div., New Hartford, CT) for 1 min followed by homogenization for 1 min (Brinkman Polytron Homogenizer, Westbury, NY). The homogenate was centrifuged at 16 000 × *g* at 15 °C for 1 h in a GSA rotor with a Sorvall RC-5B centrifuge (Kendro Laboratory Products, Newtown, CT). The supernatant was discarded, and 25 mL of 1 M NaCl in pre-extraction buffer was added to the residue and stirred 30 min at room temperature. This mixture was centrifuged at 16 000 × *g* at 15 °C for 1 h, and the supernatant was set aside. The above extraction/centrifugation procedure was repeated on the residue, and the second supernatant was combined with the first. A third extraction of the residue indicated at least 94% recovery of the PGIP activity in the first two extracts, so two extractions were routinely employed. Tests with tissue from 15-day-old fruit demonstrated that less than 10% of the tissue PGIP was lost in the pre-extraction. The cantaloupe fruit polygalacturonase inhibiting protein (CmPGIP) extract was filtered through Whatman no. 1 filter paper and centrifuged at 16 000 × *g* at 15 °C for 1 h to remove all particulate matter. The CmPGIP extract was then concentrated to 2.5 mL with a Centriprep YM-10 centrifugal filter device (Millipore Corp., Bedford, MA). The 2.5 mL of concentrated PGIP extract was exchanged into a 0.6 M sodium acetate buffer, pH 5.0, by gel chromatography with the aid of a Sephadex PD-10 column (Sigma, St. Louis, MO). The CmPGIP from the starting weight of tissue thus ended in 3.5 mL total volume.

PG and CmPGIP Activity Assays. Inhibition of PG activity by CmPGIP-containing extracts was determined by conducting the enzyme assay with and without various amounts of extract preincubated with a given PG. Approximately 5−7 nkat of PG activity were employed in each assay. Healthy cantaloupe fruit tissue extracts exhibited no detectable PG activity. Preincubations of PG alone or with various levels of CmPGIP-containing extract were carried out in a total volume of 0.45 mL at pH 5.0. This volume consisted of 150 μL of 0.05 M sodium acetate buffer containing 315 μg of BSA, 100 μL of PG in 0.05 M sodium acetate buffer, and 200 μL of various levels of CmPGIP in 0.6 M sodium acetate. Supplemental experiments showed that the final concentration of sodium acetate, 0.29 M, in the preincubation solution did not inhibit the PG-CmPGIP interaction. After preincubation at room temperature for 15 min followed by preincubation at 30 °C for 5 min, 1.35 mL of 0.133% PGA containing 700 μg/mL BSA in 0.05 M sodium acetate, pH 5.0 (pre-equilibrated to 30 ± 1 °C) were added to the preincubation mixture to initiate the reaction. Duplicate 200-μL aliquots were removed from the incubation mixture at 0, 2.5, 5.0, and 7.5 min and added to 1.2 mL of the stop buffer/derivatization reagent. The stop buffer/derivatization system, to quantify reducing groups released from sodium polygalacturonate, utilized 2-cyanoacetamide as described by Gross (*17*). D-Galacturonic acid was used as the standard to convert absorbance change to molar concentration of reducing groups formed. The R^2 value for a linear least-squares fit to the eight points collected in this discontinuous assay routinely ran 0.99 ± 0.01.

Statistical Analyses. Statistical analyses, linear regression analysis, and mean and standard deviation determinations were performed with the aid of Statistica software, version 6 (Statsoft, Tulsa, OK).

Theoretical Aspects

On the basis of the majority of enzyme−inhibitor-protein systems, a one-to-one stoichiometry is assumed in developing the theoretical models. Some of the variables used are as follows: E = amount of active target enzyme, generally expressed in units of activity; E_V = units of target enzyme remaining after addition of volume, V, of extract containing inhibitor-protein; E_{in} = units of initial target enzyme before any inhibitor-protein is added; E_{min} = units of residual apparent activity after

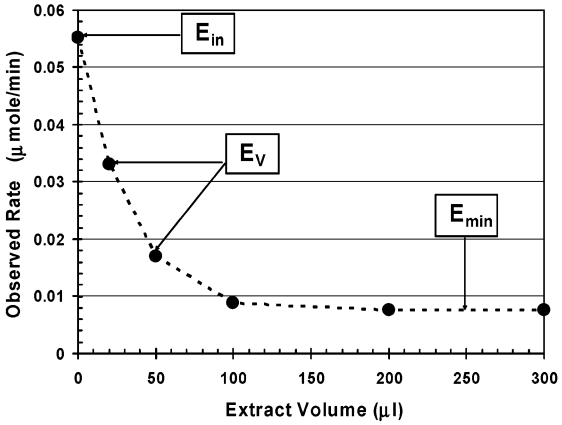

Figure 1. Typical inhibition curve of a target enzyme by its inhibitor-protein: 5.8 nkat of *P. cucurbitae* PG activity was treated with different volumes of extract from the mesocarp of 20-day postanthesis cantaloupe fruit (containing PGIP activity). Symbols in the figure are defined in the text.

all target enzyme has been inhibited; $V \equiv V_{extrct}$ = volume of extract solution containing inhibitor-protein added to the solution containing active enzyme; $V_f \equiv V_{assay}$ = final volume of the assay; $[IP]_{extrct}$ = molar concentration of inhibitor-protein in the extract; $[IP]_{free}$ = molar concentration of unbound inhibitor-protein; $[E]_{total}$ = molar concentration of total target enzyme, both free and complexed with inhibitor-protein; $[E]_{free}$ = molar concentration of uninhibited target enzyme; $[E{\cdot}IP]$ = molar concentration of inactive enzyme−inhibitor-protein complex.

In many instances, complete inhibition of the target enzyme activity does not necessarily eliminate the total observed activity as measured by a general assay such as the formation of reducing groups or the formation of free amino groups (e.g., (*20*)). This situation is illustrated in Figure 1 for the PG−PGIP system. If the assay also measures nontarget enzyme activity, the actual units of target enzyme will be obtained by adjusting the apparent activities at each point by subtracting out the residual nontarget enzyme activity. Thus, E_{in} is equal to $E'_{in} - E'_{min}$ and E_V is equal to $E'_V - E'_{min}$. The prime (′) indicates the value in the presence of "contaminating" activities in the assay; the absence of the prime indicates the actual target enzyme activity.

Model 1. A Virtually Irreversible Interaction between Enzyme and Inhibitor-Protein. The primary assumption in this model is that the association constant between enzyme and inhibitor-protein is sufficiently large so that once formed, the inactive complex does not readily dissociate. We can express the loss of enzyme activity with added inhibitor-protein as $E - V(dE/dV)$ where (dE/dV) is the amount of enzyme activity inhibited per volume of inhibitor-protein solution added. Further, if the amount of enzyme activity remaining after the addition of inhibitor is proportional to the quantity of enzyme activity at the time of addition, and using material balance, $E - V(dE/dV) = kE$, where k is a proportionality constant. Separating variables, integrating the above expression with the initial condition, $V = V_{assay} - V_{extrct}$, $E = E_{in}$, and the final condition $V = V_{assay}$, $E = E_V$, and correcting for the residual activity we get

$$\ln\frac{V_{assay}}{V_{assay} - V_{extrct}} = \frac{1}{1-k}\ln\frac{E'_V - E'_{min}}{E'_{in} - E'_{min}} \qquad (1)$$

The term $(E'_V - E'_{min})/(E'_{in} - E'_{min})$ is an expression for the fraction of target enzyme still active after addition of volume, V, of the inhibitor-protein. This quantity is also designated $(1 - \theta)$ as defined in model 2. By plotting $\ln[(E'_V - E'_{min})/(E'_{in} - E'_{min})]$ on the X axis versus $\ln[(V_{assay}/(V_{assay} - V_{extrct})]$ on the Y axis, the data are predicted to fit a straight line of intercept zero and a slope of $1/(1 - k)$. So, the volume, $V_{1/2}$, of inhibitor-protein solution needed to inhibit one-half of its target enzyme is determined by substituting −0.693 (i.e., ln(0.5)) for the abscissa value in the linear least squares equation generated for the data points. The general form of this solution is

$$V_{1/2} = V_{assay} - \frac{V_{assay}}{\exp[(\text{slope}\cdot \ln 0.5) + \text{intercept}]}$$

Model 2. An Equilibrium System of Enzyme−Inhibitor-Protein Binding. The primary assumption of this model is that there exists a reversible reaction between reactants and their inactive complex. This leads to an equilibrium state between the two reactions. Hence, for the equilibrium, $E + IP \leftrightarrow E{\cdot}IP$ the association constant at equilibrium, K_{Assoc}, can be written as

$$K_{Assoc} = \frac{[E{\cdot}IP]}{[E]_{free}[IP]_{free}}$$

If θ is the fraction of target enzyme inhibited at equilibrium, $\theta = ([E{\cdot}IP]/[E]_{total})$, then the uninhibited fraction of target enzyme is $1 - \theta = ([E]_{free}/[E]_{total})$ obtained using the enzyme activity balance, i.e., $[E]_{total} = [E]_{free} + [E{\cdot}IP]$. Further, using the balance for IP, $[IP]_{total} = [IP]_{free} + [E{\cdot}IP]$ or $[IP]_{free} = [IP]_{total} - \theta[E]_{total}$, the association constant can be reduced to $K_{Assoc} = \theta/[(1 - \theta)([IP]_{total} - \theta[E]_{total})]$. By knowing the volume of the extract and the IP concentration in the extract, $[IP]_{total}$ can be calculated using $[IP]_{total} = (V_{extrct}[IP]_{extrct})/V_{assay}$. Hence, $K_{Assoc} = \theta/(1 - \theta)[((V_{extrct}[IP]_{extrct})/V_{assay}) - \theta[E]_{total}]$. This expression can be linearized by rearrangement to obtain

$$\frac{(1-\theta)}{\theta}V_{extrct} = \frac{V_{assay}}{[IP]_{extrct}}\frac{1}{K_{Assoc}} + \left(\frac{V_{assay}}{[IP]_{extrct}}[E]_{total}\right)(1-\theta) \qquad (2)$$

Thus, a plot of $((1 - \theta)/\theta)V_{extrct}$ versus $(1 - \theta)$ should give a straight line with a slope of $(V_{assay}/[IP]_{extrct})[E]_{total}$ and an intercept of $(V_{assay}/[IP]_{extrct})(1/K_{Assoc})$. While calculating θ, correction for nonspecifically inhibited activity can be incorporated in a manner similar to model 1 to obtain $\theta = ([E]_{total} - [E]_{free})/[E]_{total} = (E'_{in} - E'_V)/(E'_{in} - E'_{min})$. Thus using a linear least-squares fit to the data, V_{extrct} can be determined by substituting the value of 0.5 for $(1 - \theta)$, i.e., 50% of the enzyme in the assay is inhibited, in the linear least squares equation and solving for "Y", i.e., $((1 - \theta)/\theta)V_{extrct}$. This is the volume of extract that inhibits one-half of the target enzyme in the assay and is identical to $V_{1/2}$ of model 1.

Comparing the Binding Affinities for Different Enzyme or Inhibitor-Protein Forms. For the PG−PGIP system, a number of isoforms of the two interacting species have been reported, together with different constants for their individual associations (*9*). Thus we extended the equilibrium model to include the ability to compare the binding affinities among enzymes from different sources with inhibitor-proteins from varied sources. Inspection of the intercept and slope terms of eq 2 suggests that additional information may be gleaned from inhibition data through the use of these terms individually or in combination.

The intercept term in eq 2 is $(V_{assay}/[IP]_{extrct})(1/K_{Assoc})$. If the inhibition of enzymes from different sources by a constant source of inhibitor-protein are compared, then V_{assay} and $[IP]_{extrct}$ are constant from one assay to the next. Thus, the ratio of the intercept values from the binding of an inhibitor-protein with two different enzymes will provide an estimate of the ratio of the enzymes' association constants with the inhibitor-protein, i.e.,

$$\frac{\text{Intercept}_1}{\text{Intercept}_2} = \frac{K_{\text{Assoc}_2}}{K_{\text{Assoc}_1}} \tag{3}$$

If the slope terms from two different inhibitor-protein sources are compared using the same enzyme at the same levels, the ratio of the slope terms predicts

$$\frac{\text{Slope}_1}{\text{Slope}_2} = \frac{[\text{IP}]_{\text{extrct}_2}}{[\text{IP}]_{\text{extrct}_1}} \tag{4}$$

that is, the ratio of the effective concentrations of inhibitor-protein is inversely proportional to their slopes since $[E]_{total}$ and V_{assay} are the same for both enzyme−inhibitor-protein systems. Further, the slope and intercept terms for a given inhibition curve can be combined in a ratio to obtain the relation

$$\frac{\text{Slope}}{\text{Intercept}} = [\text{E}]_{\text{total}} K_{\text{Assoc}} \tag{5}$$

as V_{assay} and $[IP]_{extrct}$ cancel out. Thus, it is also possible to compare the ratio of binding constants of inhibitor-proteins from two sources by using identical amounts of the same target enzyme with each of the two inhibitor-protein sources. Note that the comparison of the binding affinities for two different sources of PGIPs is based on the assumption that the crude extract has only one isoform and reactions due to the presence of other isoforms are negligible.

Results

Comparison of the Two Models for Calculating Inhibitor-Protein Activity. The validity of using either model 1 or 2 was compared for over 30 different PGIP-containing extracts obtained using the methods described in materials and methods. Figures 2 and 3 show examples of the plots generated using the experimental data and the coordinates of eq 1 and eq 2, respectively. A percent difference was expressed by subtracting the estimate by eq 2 from that by eq 1 and dividing the difference by their average. The average percent difference for the 30 samples was 1.8% ± 7.6% (SD). Diluting a PGIP-containing extract 4-fold and quantifying its PGIP content by both eqs 1 and 2 gave an estimated PGIP content for the 4-fold dilution of 24% ± 3% ($n = 4$) that of the stock solution.

Validity of the Two Models Evaluated with Literature Data. Next, we extended the validation of eqs 1 and 2 to include the use of data from the literature. As shown in Table 1, both models yielded results in reasonable agreement with the reported values. However, eq 1 was more sensitive to errors in the determination of $(E'_{in} - E'_{min})$ than eq 2. Utilizing assays for the PG−PGIP system, a 20% error in determination of $(PG'_{in} - PG'_{min})$ resulted in a 10−15% error in the estimate of inhibitor activity per volume unit by eq 1. The same error in $(PG'_{in} - PG'_{min})$ yields <5% error in activity per volume unit by eq 2.

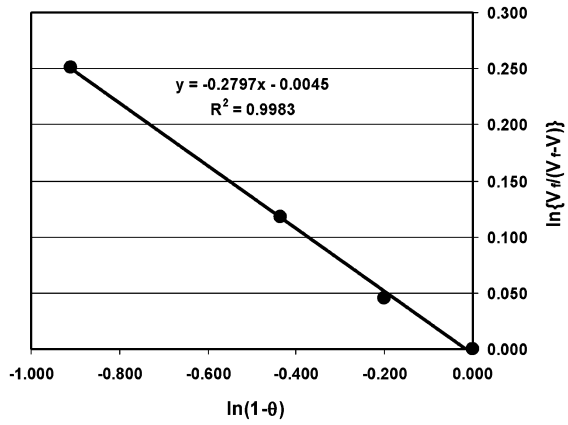

Figure 2. Application of model 1 to quantify inhibitor-protein activity. *A. niger* PG was treated with various volumes of PGIP-containing extract from seed cavity material of 5-day postanthesis cantaloupe fruit. The amount of PG activity remaining after addition of each volume of inhibitor was then measured. Data were plotted according to eq 1.

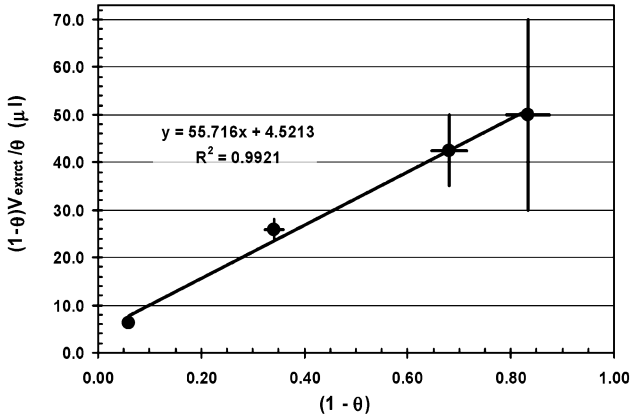

Figure 3. Application of model 2 to quantify inhibitor-protein activity. *F. solani* PG was treated with various volumes of PGIP-containing extract from seeds of 15-day postanthesis cantaloupe fruit. The level of PG activity remaining after addition of each volume of inhibitor was then measured. Data were plotted according to eq 2. Error bars indicate the obtainable values with 5% variation in $E_V - E_{min}$.

Table 1. Quantification of PGIP Levels Using Data from the Literature

system	model 1 eq 1	model 2 eq 2	literature value
A. niger vs pear PGIP I	0.02[a]	0.02[a]	0.02[a] (*28*)
A. niger vs pear PGIP II	0.039[a]	0.037[a]	0.035[a] (*28*)
B. cinerea vs apple PGIP	0.93[b]	0.95[b]	0.96[b,c] (*29*)
C. lindemuthianum vs bean PGIP	0.13[a]	0.15[a]	0.13[a,c] (*13*)

[a] Micrograms of PGIP. [b] Units of PGIP activity. [c] Interpolated from a figure in the reference.

Evaluation of the Interaction between PGs and PGIPs from Different Sources. To evaluate the validity of model 2 in comparing the ratios of binding affinities, we used the interactions between PGs from two fungal sources and PGIPs from two stages of fruit development. The inhibition curves are shown in Figure 4A and are plotted according to eq 2 in Figure 4B. According to eq 4, the ratios of the effective concentrations of PGIP in 5-day-old fruit to that in 35-day-old fruit versus *F. solani* PG yields a value of 5 and versus *P. cucurbitae* PG yields a value of 1. These two ratios were confirmed by comparing the individually determined concentrations through the applications of eq 1 to the inhibition data. The data are consistent with the quantity of PGIP inhibitory

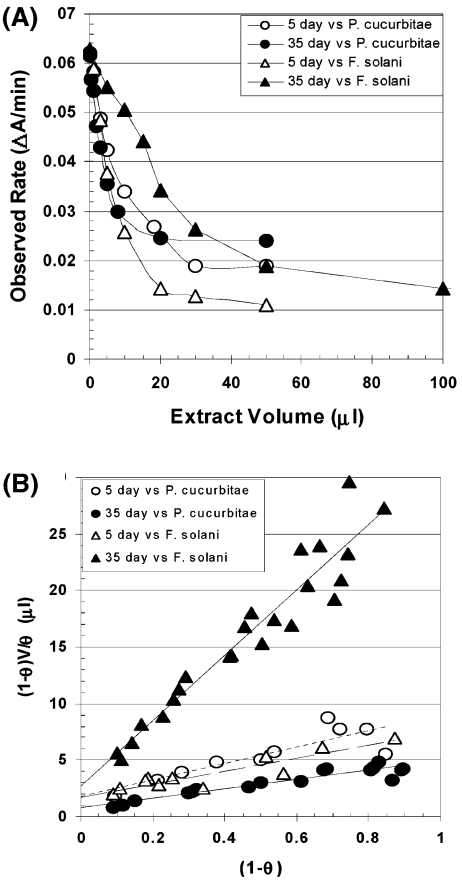

Figure 4. Inhibition of fungal pathogen PGs with PGIP from cantaloupe fruit at different stages of development. (A) Inhibition curves for the interaction of PGIP from seed cavity (placental) tissue of 5-day or 35-day (ripe) postanthesis cantaloupe fruit versus PGs from *P. cucurbitae* or *F. solani*. Fixed levels of PG activity were treated with increasing volumes of tissue extract, and the remaining activity was measured. (B) The data of part A, together with data from additional measurements, were plotted according to eq 2.

activity versus *P. cucurbitae* PG remaining constant from day 5 to day 35 while it decreases to about one-fifth of its 5-day level versus *F. solani* PG. Comparison of the intercept values for *P. cucurbitae* PG and *F. solan*i PG with 5-day-old placental material yields a ratio of association constants of 1.0. With 35-day-old placental material, the association constant with *P. cucurbitae* PG is ~3.3 times that with *F. solani* PG. Thus, the association constants for the interactions of the two PGs with PGIP from 5-day-old placental material are similar in magnitude.

The slope and intercept ratio for PGIP from each of the two ages of fruit versus F. *solani* PG were calculated according to eq 5. The slope and intercept ratio for PGIP from 5-day-old placenta was then divided by the slope and intercept ratio for 35-day-old placenta to give a value of 0.32. This is consistent with the association constants of the PGIPs from the two ages of fruit being similar in magnitude. The same ratio determined for the two PGIP sources versus *P. cucurbitae* PG gives a value of 0.8 and suggests that the association constants of the PGIPs from the two ages of fruit are roughly the same.

Extension of the Comparison of Association Constants. Next, we extended the comparison of the ratios of association constants to other PG-PGIP systems using limited data from the literature. As shown in Table 2, this comparison showed poor and highly variable agreement between the reported values and the calculated

Table 2. Comparison of Ratios of Association Constants

system	K_{a_1}/K_{a_2} est by eq 3	K_{a_1}/K_{a_2} direct determ	ref
bulk bean PGIP vs *F. moniliforme* PG (1)[a] or *F. moniliforme* PG expressed in yeast (2)[a]	0.47	5.0	(*30*)
bean PGIP2 vs *F. moniliforme* PG (1) or *A. niger* PG (2)	0.17	0.021	(*31*)
bulk bean PGIP vs *A. niger* PG (1) or *F. moniliforme* PG (2)	1.30	0.92	(*32*)

[a] The number refers to the respective association constant as written in column headers two and three.

values. However, these variable results can be attributed in each case to limitations in the amount of inhibition data that were applicable for the estimates by eq 3 (usually there were only three data points in the range appropriate to fit eq 2). Since the relationship depends on extrapolation to the *Y*-axis intercept, the accuracy of estimates employing eq 3 will depend significantly on the accuracy of the extrapolation and hence on the accuracy of the experimental points. To evaluate the role of experimental uncertainty on the extrapolation and the final outcome of the binding constant values, we computed the possible errors in the *Y*-axis values based on errors incurred in measuring E_V. These results showed that at very low fractional binding of the enzyme with inhibitor-protein (low θ values), the predicted values using model 2 are very sensitive to experimental variation in the enzyme activity, E_V, values. As shown by the error bars in Figure 3, 5% (i.e., with 95% confidence interval) variation in the E_V resulted in 5% error in $(1 - \theta)$ values and 42% error in the $(1 - \theta)\cdot V/\theta$ at $\theta < 0.2$. At θ values >0.4, a 5% variation in E_V resulted in less than 5% error in $(1 - \theta)\cdot V/\theta$. The scatter in the data in Figure 4B at $\theta < 0.2$ could also be attributed to the same behavior.

Discussion

The interaction of an enzyme from one of several classes of hydrolytic enzymes with its specific inhibitor-protein results in a complex that is inactive or of greatly depressed activity.The two simple models outlined above are of great importance when quantifying an inhibitor-protein activity, especially in a crude system. Although both models provided similar activity values, the first model allows one to use the "enzyme only" data point in the plot, so it offers one additional point to the graph than does the second model for a given set of assays. However, the second model is applicable to all enzyme−inhibitor-protein systems regardless of their strength of interaction. The utilization of combinations of enzyme sources and inhibitor-protein sources allows the extraction of considerably greater amounts of information than merely the individual inhibitor curves when the data are treated by the equilibrium-derived relations given by eqs 3−5. Their potential utility was demonstrated by applying them to inhibition data between PGs from two fungal sources and PGIPs from two stages of fruit development (Figure 4). The data of Figure 4A raise the question as to the explanation for the apparent diminished effectiveness of PGIP between 5-day-old fruit and 35-day-old fruit versus PG from *F. solani*, whereas during this same period of fruit development, the PGIP retains its effectiveness against PG from *P. cucurbitae*. The data suggest up-regulation of a different form of the inhibitor in the mature fruit. However, presented as it is in Figure 4A, the data cannot discriminate between two scenarios.

In one scenario, the 35-day form of PGIP is synthesized at levels commensurate with the form produced at 5 days, but the 35-day form binds with a lower association constant. In the second scenario, the 35-day form of PGIP does not bind and inhibit *F. solani* PG at all, and thus, inhibition of the *F. solani* PG is effected by residual levels of the 5-day form of the inhibitor. On the basis of the results by treatment of the inhibition data with the equilibrium model, however, it is possible to eliminate the first scenario. The predominant form of PGIP produced by 5-day-old cantaloupe fruit binds to *F. solani* and *P. cucurbitae* PGs with roughly equal affinities. Between 5 and 35 days, this first form of PGIP is apparently down-regulated while a second form is apparently up-regulated. This second form binds to *P. cucurbitae* PG with an affinity similar to the form from 5-day-old fruit. Conversely, this second, or 35-day form of PGIP binds very weakly or not at all to *F. solani* PG. Thus, it would appear that the inhibition of *F. solani* PG from placental material of 35-day-old fruit is accomplished by greatly reduced levels of the PGIP form predominantly expressed at 5 days. Using *F. solani* as the test PG source, the relative amounts of these two forms of PGIP at 35 days are estimated to be ∼15−25% of the 5-day form and ∼75−85% of the 35-day form.

The use of the equilibrium model and its attending relationships to obtain estimates of the ratios of equilibrium constants or inhibitor quantities in crude systems or for limited quantities of pure materials remains to be rigorously documented. We have been unable to find sufficient data in the literature for specific enzyme−inhibitor-protein systems that couple inhibition data with results for their binding as measured by established equilibrium binding measurements such as calorimetry (*21*) or surface plasmon resonance (*22*) to adequately test these relationships of the equilibrium model. A successful comparison of the analysis of enzyme inhibition as outlined in this manuscript with equilibrium constants measured by accepted physical methods must ultimately provide the rigorous test of the potential utility of eq 2 and its attending relationships.

While developing the models, we assumed a one to one binding between the enzyme and inhibitor-protein species as well as homogeneous populations of each. However, a number of isoforms of both interacting species have been reported together with different association constants (*1, 9*) for their binding. A crude extract and, indeed, many purified preparations will likely be composed of more than one isoform of each species at differing concentrations. To better understand the competitive binding of each isoform, the model has to be extended to include multiple reactions. To establish the concepts, experiments have to be performed with known mole fractions of different isoforms to assess their relative impacts. Nevertheless, the application of the relationships derived herein offers the investigator a route to preliminary information about the system under investigation. Extension of these models to other systems to include other PG and PGIP sources such as melons (*23*), apples (*24*), tomatoes (*25*), soybean (*26*), and maize (*27*) will have significant impact on understanding the role of enzyme−inhibitor-protein systems in the growth and development of fruits and vegetables. Further, the influence of impurities on the binding characteristics and the alterations in the binding affinities between inducible forms has to be performed (*10, 15*). Inclusion of these parameters into the models could provide a better quantitative method to estimate the inhibitor-protein activities and also provide information on the important stage-specific alterations in the expression of inhibitor-proteins during growth and development. This could lead to minimal genetic manipulation of dominant inhibitory factors while engineering new naturally enhanced plant disease-resistance systems or to better understanding the process of evolution.

In conclusion, this report demonstrates that the irreversible binding approximation (the first model) can be used for inhibitor-protein activity determination, and the reversible-binding model (the second model) can be used for both activity determination and for comparison of binding constants among varied enzyme and inhibitor-protein sources.

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